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Molecular Cloning of the Six mRNA Species of Infectious Hematopoietic Necrosis Virus, a Fish Rhabdovirus, and Gene Order Determination by R-Loop Mapping†

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Plasmids carrying cDNA sequences to the mRNA species of infectious hematopoietic necrosis virus were constructed and cloned into *Escherichia coli*. Characterization of 21 cloned plasmids by hybridization to mRNA blots identified sets of plasmids with homology to each of the six viral mRNA species. R-loop mapping with these cDNA plasmids determined that the gene order on the infectious hematopoietic necrosis virus genome is (3′)N-M1-M2-G-NV-L(5′).

Infectious hematopoietic necrosis virus (IHNV) is a salmonid rhabdovirus with a single-stranded RNA genome of ca. 10,900 bases (11). The virion is comprised of five proteins (9, 14, 19): a nucleocapsid protein, N; a surface glycoprotein, G; two matrix proteins, M1 and M2; and a polymerase, L. Although IHNV resembles the mammalian rhabdovirus prototypes, vesicular stomatitis virus, and rabies, there are some significant differences, including the encoding of a nonvirion protein which has been designated the NV protein (11).

The slow growth and relatively low yield of IHNV in tissue culture (15) made it necessary to obtain cloned genetic material for further studies of the molecular biology of the virus. We present here the construction of cDNA clones carrying sequences from each of the six mRNA species of IHNV. These clones were used in hybrid selection studies to determine coding assignments for the viral mRNA species (11) and in R-loop analyses to obtain a physical map of the viral genome.

MATERIALS AND METHODS

Cells and virus. IHNV was propagated in a chinook salmon embryo cell line (CHSE-214) as described previously (11).

Cloning of viral mRNA species. Polyadenylated RNA was isolated from IHNV-infected CHSE-214 cells as described previously (11). For use as a cloning template, this RNA was passed twice over an oligodeoxythymidylic acid-cellulose column to remove all detectable host cell ribosomal RNA. The preparation of double-stranded cDNA was carried out by the procedure of Land et al. (13). This procedure includes 4 mM sodium PP_i in the first-strand cDNA reaction to prevent the formation of the terminal hairpin loop and eliminates the need for S1 nuclease digestion.

Briefly, 20 μg of polyadenylated RNA was reverse transcribed to synthesize 3.9 μg of single-stranded cDNA in a reaction containing the RNA template, an oligodeoxythymidylic $acid_{(12-18)}$ primer, placental RNase inhibitor (Enzo Biochemicals, Inc.), and reverse transcriptase (Life Science Div., The Mogul Corp.). Tails of ca. 20 dCMP residues were added to this single-stranded cDNA with terminal deoxynucleotidyl transferase, and these molecules were rendered double stranded in a second reverse transcriptase reaction containing oligodeoxyguanylic $acid_{(12-18)}$ (Collaborative Research, Inc.) as the primer. Nicks or gaps in these double-stranded molecules were filled by incubation with Klenow enzyme (18), and the products of this reaction were tailed with dCMP residues.

The plasmid vector, pUC8, was cleaved with the restriction endonuclease *Pst*I (Bethesda Research Laboratories), and deoxyguanylic acid tails of ca. 15 residues were added. The deoxyguanylic acid-tailed vector and deoxycytidylic acid-tailed cDNA were annealed at a molar ratio of 1:1 (13), and this DNA was used to transform two host strains of *Escherichia coli* K-12, JM103 (22), and C600 SC181 (2). Transformation was carried out with freshly prepared competent cells by the calcium chloride shock method (17). Transformants were plated on LB agar (18) containing 150 μg of ampicillin per ml. The transformation efficiencies were 51 and 158 ampicillin-resistant transformants per ng of reannealed DNA for JM103 and SC181, respectively. The control transformation efficiency was 5×10^3 transformants per ng of uncleaved pUC8 for both strains.

Preparation of cDNA probe. The probe for viral-specific sequences was ^{32}P-labeled cDNA to the IHNV genome RNA. Probe synthesis was carried out at 42°C for 105 min in a 50-μl reaction containing 1 μg of fragmented viral genome RNA; 0.5 μg of calf thymus random primer fragments (28); 50 mM Tris-hydrochloride (pH 8.3); 40 mM $MgCl_2$; 0.4 mM dithiothreitol; 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, and 0.01 mM [^{32}P]dCTP (specific activity, 800 Ci/mmol; New England Nuclear Corp.); and 40 U of reverse transcriptase (Life Science Div.). After synthesis the reaction was adjusted to 0.6 N NaOH and incubated at 37°C for 30 min. The reaction was then neutralized by adding HCl to 0.6 N and Tris-hydrochloride (pH 8.1) to 200 mM. This mixture was passed over a 5-ml column of Sephadex G-50, and fractions containing incorporated radioactivity were pooled, adjusted to 0.3 M potassium acetate, and precipitated with 2.5 volumes of ethanol. This probe typically had a specific activity of 2×10^7 cpm/μg.

Colony blots. Transformants were screened for viral sequences by the procedure of Taub and Thompson (27), in which fresh colonies were replicated onto sterile Whatman 541 filter paper and washed successively in NaOH, lysozyme, proteinase K, and phenol-chloroform-isoamyl alcohol (25:41:1). Filters were hybridized with 2×10^6 to 5×10^6

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cpm of ^{32}P-labeled cDNA probe (see above) as described previously (27).

Isolation of plasmid DNA. Small-scale plasmid preparations (1 to 2 μg) were made by a modification of the alkaline lysis procedure (4). Fresh colonies of each transformant were scraped from plates with a toothpick and dispersed vigorously in 40 μl of a lysing buffer composed of 0.5% sodium dodecyl sulfate, 50 mM NaOH, 5 mM EDTA, and 2% Ficoll. These mixtures were incubated for 30 min at 68°C. Sucrose and bromphenol blue were added to 5 and 0.002%, respectively, and the samples were loaded onto a horizontal 0.7% agarose gel in Tris-acetate buffer (0.72 M Tris-hydrochloride, 0.1 mM acetate, 20 mM EDTA, [pH 7.9]). After electrophoresis for 12 to 16 h at 25 V, the gel was stained with ethidium bromide and examined with a UV transilluminator (Fotodyne, Inc.).

Large-scale isolation of plasmid DNA was carried out by the boiling method of Holmes and Quigley (10).

Determination of cloned viral sequence size. The restriction endonuclease *Pst*I (Bethesda Research Laboratories) was used to cleave 300 ng of each purified plasmid. The released cloned insert DNA was separated from the plasmid vector DNA by electrophoresis on a vertical 7.5% acrylamide gel with a 3.75% acrylamide stacking gel. The Laemmli gel system (12) was used with the exception that sodium dodecyl sulfate was omitted from all buffers. Electrophoresis was carried out at 30 mA through the stacking gel and 50 mA through the lower gel. After electrophoresis, the gel was stained with ethidium bromide, and photographs of the UV-illuminated gel were made with Polaroid type 47 film. The sizes of the cloned inserts were determined by comparison with *Hin*fI and *Hae*III pBR322 restriction fragment size standards.

Preparation of nick-translated probes. Nick translation of cloned plasmids was carried out as described previously (18, 24) in 10-μl reactions containing 200 ng of plasmid DNA. Nick-translated probes had a specific activity of 2×10^7 to 5×10^7 cpm per μg.

DNA blot hybridizations. Cloned plasmids were cleaved with the restriction endonuclease *Pst*I (Bethesda Research Laboratories), and the cloned inserts were separated from vector DNA by electrophoresis on 0.7% agarose gels in Tris-acetate buffer (see above). The DNA was alkaline denatured, transferred to a nitrocellulose membrane by the Southern blot method (18, 26), and baked onto the filter for 2 h at 80°C in vacuo. Hybridization of the blots was as described previously (18) with the exception that both prehybridization and hybridization were carried out in 50% formamide at 42°C. Each hybridization included 2×10^6 to 10×10^6 cpm of ^{32}P-labeled cDNA probe or nick-translated probe. After hybridization, blots were washed as described previously (18) and exposed to Kodak X-Omat AR X-ray film with a Cronex Lightening-Plus intensifying screen (Du Pont Co.) at −70°C.

mRNA blot hybridization. Hybridization of ^{32}P-labeled, nick-translated probes to blots of viral mRNA were carried out by the procedures of Thomas (29). Polyadenylated RNA from IHNV-infected cells was resolved into five bands by glyoxal gel electrophoresis (11, 21) and transferred to a nitrocellulose membrane as described previously (29). The blot was baked for 2 h in vacuo at 80°C and cut into strips, each containing one gel lane with 2 μg of RNA. The strips were boiled for 5 min in 20 mM Tris-hydrochloride (pH 8.0) to remove the glyoxal adducts and hybridized individually as described previously (29) with 5×10^6 to 10×10^6 cpm of ^{32}P-nick-translated probes. After hybridization, blots were washed as described previously (29) and exposed to X-ray film as described above.

R-loop mapping. IHNV genomic RNA for R-loop mapping was prepared as described previously (11). Cloned IHNV cDNA plasmids were prepared as described above for large-scale isolation and linearized by cleavage with *Nde*I (Bethesda Research Laboratories). Formamide (Bethesda Research Laboratories) was deionized by treatment with AG 501-X8 mixed bed ion-exchange resin (Bio-Rad Laboratories). R-loops were formed as previously described (5) with the following modifications. Linearized plasmid DNA (100 ng) in 18 μl of 78% formamide–5 mM EDTA was denatured at 80°C for 10 min. IHNV RNA (100 ng) was added to the denatured DNA, the solution was adjusted to 20 μl of 70% formamide–300 mM NaCl–4.5 mM EDTA, and R-loops were formed by incubation at 50°C for 12 to 16 h. Samples (4 μl) of the R-loop reaction mixture were spread by the urea-formamide method (23). The hyperphase (40 μl) consisted of 4 M urea, 80% formamide, 5 mM EDTA, and cytochrome *c* at a concentration of 40 μg/ml. The hypophase (20 ml) was 50% formamide. The DNA-protein film was adsorbed to a Parlodion-coated grid, stained with uranyl acetate, and rotary shadowed with platinum-palladium. Grids were examined with a Zeiss EM-10A electron microscope operating at 40 kV. Molecular lengths were measured by a calculator-driven digitizer on photographic prints enlarged to a final magnification of 136,000. DNA molecules of known sequence were used as length standards. Double-stranded DNA and RNA · DNA hybrid duplexes had identical contour lengths under the spreading conditions used in these experiments. IHNV RNA lengths were converted to nucleotides, using a unit length of 10,900 nucleotides and correcting for RNA in RNA · DNA hybrid duplexes.

RESULTS

Preparation of cloned plasmids carrying viral cDNA. The mRNA species of IHNV have been isolated from IHNV-infected salmon cells and resolved electrophoretically into five bands which range in size from 1.95×10^5 to 2.26×10^6 (11). This RNA was used as a template for the preparation of cDNA clones carrying IHNV mRNA sequences. Briefly, polyadenylated RNA from IHNV-infected cells was copied into cDNA with reverse transcriptase and an oligodeoxythymidylic acid primer. Analysis of the products of the first-strand cDNA reaction by alkaline gel electrophoresis showed four distinct species corresponding in size to full-length copies of the mRNA bands 2, 3, 4, and 5 (data not shown). This single-stranded cDNA was copied into double-stranded molecules, polydeoxycytidylic acid tailed with terminal transferase, and annealed into the *Pst*I site of the plasmid vector pUC8. Two *E. coli* K-12 host strains, JM103 and SC181 (C600), were transformed, and ca. 800 ampicillin-resistant transformants were isolated from each. Colony-blotted DNA of ca. 90% of the transformants hybridized with a viral genome cDNA probe. Small-scale plasmid preparations (1 to 2 μg) showed that ca. 10% of the transformants had relatively large plasmids. The cDNA inserts of purified plasmids from 21 of these transformants were released by *Pst*I cleavage and determined by electrophoresis to range in size from 155 to 640 base pairs (Table 1). Southern blot analyses of *Pst*I-cleaved plasmids showed that the insert DNA hybridized strongly with a viral genome cDNA probe (data not shown), reconfirming that the cloned sequences were virus specific.

mRNA blot analyses. Characterization of the cloned plasmids required that we identify the specific viral mRNA

TABLE 1. Viral insert size and mRNA specificity for cloned plasmids

Cloned plasmid	Insert size (base pairs)	mRNA specificity[a]	Viral gene[b]
pL232	640	1	L
pL262	640	1	L
pG480	440	2	G
pN512	370	3	N
pN933	320	3	N
pN421	350	3	N
pN154	405	3	N
pN156	400	3	N
pN419	460	3	N
pN125	450	3	N
pN144	450	3	N
pM1163	425	4	M1
pM1420	155	4	M1
pM219	505	4	M2
pM2173	265	4	M2
pM211	510	4	M2
pM2112	515	4	M2
pM2132	510	4	M2
pNV58	420	5	NV
pNV137	395	5	NV
pNV711	220	5	NV

[a] Determined by mRNA blot hybridizations (Fig. 1).
[b] Determined for all genes but L by hybrid selection and in vitro translation. The L gene was assigned tentatively to mRNA 1 by molecular weight correlation (11).

TABLE 2. Cross-hybridization within sets of plasmids with cDNA to the same viral mRNA species

Plasmids	pNV58	pL262	pN144	pN419	pM219	pM1163
A. mRNA band 5						
pNV58	+					
pNV137	+					
pNV711	+					
B. mRNA band 1						
pL262		+				
pL232		+				
C. mRNA band 3						
pN512			+	−		
pN933			+	−		
pN421			+	−		
pN154			+	−		
pN156			+	−		
pN125			+	−		
pN144			+	−		
pN419			−	+		
D. mRNA band 4						
pM219					+	−
pM2173					+	−
pM2112					+	−
pM2132					+	−
pM1163					−	+
pM1420					−	+

species which was complementary to each cDNA insert. This was carried out by probing nitrocellulose blots of the mRNA electrophoretic pattern with ^{32}P-labeled probes made by nick translation of each of the 21 purified plasmids. Examples of plasmids which hybridized specifically to each of the five mRNA bands are shown in Fig. 1.

Identification of the single mRNA band which hybridized with each plasmid DNA probe was made by comparison of the blot autoradiogram with a marker lane of all five mRNA bands (Fig. 1a). However, the proximity of mRNA bands 2 and 3 made it difficult to distinguish hybridization to mRNA band 2 with certainty. Therefore, a double hybridization was carried out with two probes together, pG480 and pN144 (Fig. 1g). The presence of two bands of hybridization confirmed that cDNA clones with sequences from both mRNA bands 2 and 3 had been isolated (Table 1). Of the 21 plasmids tested,

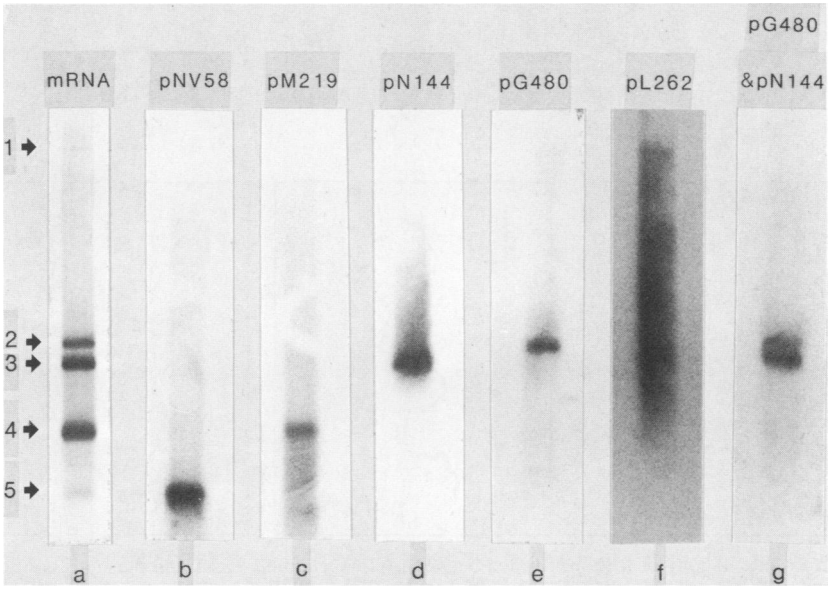

FIG. 1. mRNA blot hybridization analyses of cloned plasmids. IHNV mRNA isolated from infected salmon cells (11) was resolved by glyoxal gel electrophoresis, blotted onto a nitrocellulose membrane, and hybridized with ^{32}P-labeled probes made by nick translation of cloned plasmids. Lane a is an autoradiogram of the IHNV ^{3}H-mRNA electrophoretic profile identical to those used to prepare the blots. Numbers to the left of lane a designate IHNV mRNA bands 1 through 5. Lanes b through g are autoradiograms of blots after hybridization with probes of the following plasmids; b, pNV58; c, pM219; d, pN144; e, pG480; f, pL262; g, pG480 and pN144.

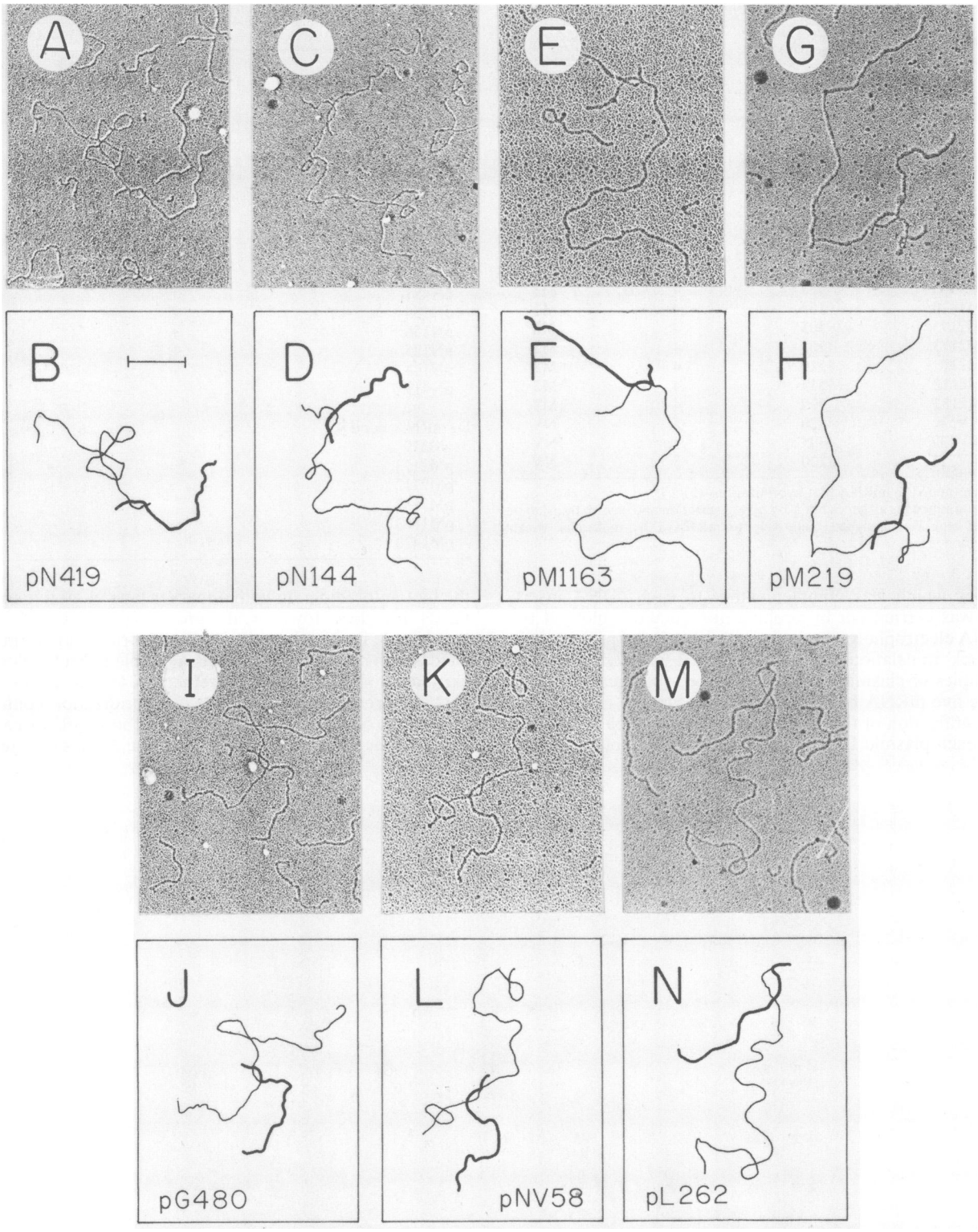

FIG. 2. Analysis by electron microscopy of R-loops formed between IHNV genomic RNA and plasmids containing cloned IHNV cDNA sequences. Interpretive drawings of each R-loop are shown below each photograph in which the thin line is IHNV RNA (or the displaced, single-stranded DNA in the R-loop) and the thick line is double-stranded plasmid DNA (or the RNA · DNA hybrid duplex in the R-loop). The bar in panel B represents 1,000 base pairs. R-loops were formed between IHNV RNA and pN419 (A and B), pN144 (C and D), pN1163 (E and F), pM219 (G and H), pG480 (I and J), pNV58 (K and L), and pL262 (M and N).

TABLE 3. Measurements of single R-loops and location of hybridizations on the viral genome

Plasmid	Measurements of[a]:			Location of[b]:	
	Short end	Loop	Long end	N-ward nucleotide	L-ward nucleotide
pN419		490 ± 50	10,410 ± 500		490 ± 50
pN144	960 ± 110	440 ± 40	9,500 ± 450	960 ± 110	1,400 ± 150
pM1163	1,520 ± 220	410 ± 60	8,970 ± 390	1,520 ± 220	1,930 ± 285
pM219	2,020 ± 250	530 ± 60	8,350 ± 470	2,020 ± 250	2,550 ± 310
pG480	4,180 ± 190	400 ± 40	6,320 ± 240	4,180 ± 190	4,580 ± 230
pNV58	4,430 ± 240	450 ± 40	6,020 ± 270	4,430 ± 240	4,880 ± 280
pL262		710 ± 70	10,190 ± 500	10,190 ± 500	10,900 ± 570

[a] In nucleotides (± standard deviation) assuming 10,900 nucleotides as the complete length of the genome.
[b] Nucleotide position (± standard deviation) of R-loop boundaries from 0 (N-ward end) to 10,900 (L-ward end).

2 hybridized to mRNA 1, 1 hybridized to mRNA 2, 8 hybridized to mRNA 3, 7 hybridized to mRNA 4, and 3 hybridized to mRNA 5.

Cross-hybridization studies. It was of interest to examine the extent of cross hybridization between the cDNA inserts of these cloned plasmids. This was done by hybridizing blots of *Pst*I-restricted, electrophoretically resolved plasmid DNA with probes made by nick translation of other cloned plasmids. As was expected, there was strong hybridization of every probe to the linear pUC8 DNA. However, hybridization to the released insert DNA was dependent on the specific cDNA of each probe. In no case was there hybridization between inserts which had been identified previously as carrying cDNA to different mRNA bands (data not shown).

Within sets of plasmids which hybridized to the same mRNA band there was considerable homology. Salient results of the cross-hybridization studies are compiled in Table 2. The inserts of the three plasmids which carried cDNA to mRNA band 5 all showed specific cross-hybridization. In like manner, the inserts of the two mRNA band 1 plasmids hybridized to each other. Of the eight plasmids with cDNA to mRNA band 3, seven of the inserts did cross-hybridize, and one insert, pN419, did not. Hybridization selection studies (11) have shown that both pN419 and the other mRNA band 3 plasmids are complementary to the mRNA for the viral N protein.

Analysis of the seven plasmids which hybridized to mRNA band 4 indicated that there were two mutually exclusive subsets of homologous cDNA plasmids (Table 2D). Hybrid selection studies (11) revealed that mRNA band 4 was comprised of two distinct comigrating mRNA species which encode the viral M1 and M2 proteins. A subset of five cross-hybridizing mRNA 4 plasmids carried cDNA to the mRNA for M2, and a subset of two mRNA 4 plasmids carried cDNA to the mRNA for M1 (Table 1).

Heteroduplex mapping. Once cDNA clones were identified which carried sequences from each of the six viral genes, it was possible to determine the physical order of the genes on the viral genome by R-loop mapping with viral genomic RNA.

The first set of R-loops was prepared by annealing viral genomic RNA with a single cloned plasmid carrying cDNA to each viral gene. Plasmids pL262, pG480, pN144, pM1163, pM219, and pNV58 were used to locate genome sequences of the L, G, N, M1, M2, and NV genes, respectively. In addition, plasmid pN419 was included since it failed to cross-hybridize with the other plasmids carrying cDNA from mRNA 4 (Table 2C). Examples of the R-loops observed are shown in Fig. 2. Measurements of 15 to 20 R-loops formed with each plasmid were used to locate the region of hybridization along the viral genome. The percentage of the full-length genome RNA on both sides of each R-loop was determined and converted to nucleotide values, assuming 10,900 bases as the full length of the genome (11) (Table 3).

Both of the inserts of the N gene plasmids, pN144 and pN419, hybridized near the end of the genome, within the terminal 10% of the complete genome length. The L gene plasmid, pL262, also hybridized within the terminal 7% of the genome length. Since the L and N insert sequences did not cross-hybridize, we concluded that these clones were hybridizing to opposite ends of the genome. Thus, we were able to orient the genome with respect to N-ward and L-ward ends.

These data alone were not sufficient to determine the gene order because the two ends of the genome were indistinguishable in the electron micrographs of single R-loops. Therefore, to determine the relative positions of the viral genes, double R-loop reactions were carried out in which pairs of cloned plasmids were annealed simultaneously to genomic RNA. Examples of genomic RNA with two R-loops are shown in Fig. 3, and appropriate measurements are given in Table 4. The distances between pairs of R-loops formed between the pN144 and pM1163, mP219, pG489, or pNV58 indicate that the N, M1, M2, G, and NV genes are clustered, in that order, within the N-ward half of the genome. The long distance between double R-loops formed with pL262 and pM1163 indicated that the L gene insert did hybridize to the end of the genome distal from the other five genes. Thus, the order of the genes from the N-ward to the L-ward ends of the genome was concluded to be N-M1-M2-G-NV-L.

DISCUSSION

We have presented the construction and characterization of cDNA clones carrying sequences from each of the six mRNA species of IHNV. The strategy for constructing the clones involved the use of an oligodeoxythymidylic acid primer, which would hybridize to the 3′ polyadenylated tail of the viral mRNA species and prime cDNA synthesis in the 5′ direction. Theoretically, all the cloned plasmids should contain the intact 3′ terminal sequence of the corresponding viral mRNA and various lengths of sequence in the 5′ direction. Although alkaline gel analysis of the first-strand cDNA products indicated the synthesis of complete copies of mRNA bands 2, 3, 4, and 5 (data not shown), there were no full-length sequences found upon analysis of the cloned plasmids. We hypothesize that this occurred because of the lack of size selection before annealing and the preferential annealing of shorter double-stranded cDNA molecules into the vector. Thus, the clones we have described carry partial sequences of each viral mRNA and should include the 3′-terminal sequences.

The plasmids from a set of 21 transformants were characterized by hybridization to blots of viral mRNA, and the

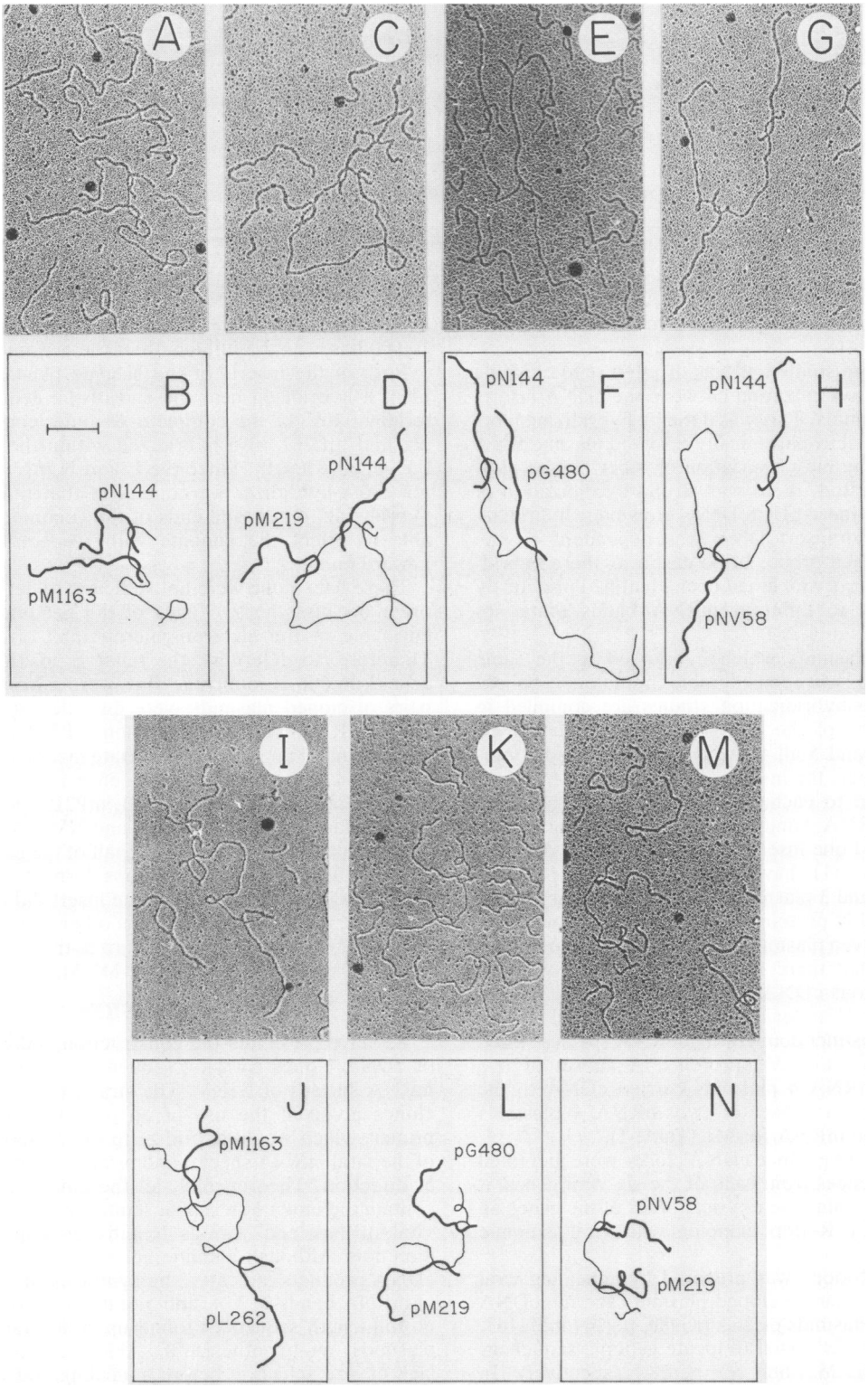

FIG. 3. Analysis by electron microscopy of R-loops formed between IHNV genomic RNA and pairs of plasmids containing cloned IHNV cDNA sequences. See the legend to Fig. 2 for details. The bar in panel B represents 1,000 base pairs. R-loops were formed between IHNV RNA and the following pairs of plasmids: pN144 and pM1163 (A and B), pN144 and pM219 (C and D), pN144 and pG480 (E and F), pN144 and pNV58 (G and H), pM1163 and pL262 (I and J), pM219 and pG480 (K and L), and pM219 and pNV58 (M and N).

TABLE 4. Gap measurements for double R-loops

Plasmids	Gap[a]
pN144 and pM1163	440 ± 30
pN144 and pM219	1,140 ± 110
pN144 and pG480	2,710 ± 410
pN144 and pNV58	3,260 ± 400
pM1163 and pL262	7,630
pM219 and pG480	1,470 ± 70
pM219 and pNV58	2,160 ± 100

[a] In number of nucleotides (± standard deviation) between adjacent boundaries of the two R-loops.

specific mRNA species which was complementary to each cloned sequence was determined (Table 1). The number of clones carrying cDNA to each mRNA band generally reflected the relative abundance of that mRNA in the total mRNA preparation used as the template for cloning. Thus, the majority of the clones carried cDNA to mRNA bands 3 and 4, which had the highest molar ratios of the mRNA species in the total preparation (11).

Cross-hybridization studies showed that the majority of cloned sequences which hybridized to the same mRNA band also hybridized with each other (Table 2). This indicated that the same or significantly overlapping regions of the mRNA sequence had been cloned in most cases, as would be expected if each contained the 3′ mRNA terminus. The only exception to this was plasmid pN419, which hybridized to mRNA band 3 but did not hybridize with any of the other seven clones exhibiting hybridization to mRNA band 3 (Table 2C). R-loop mapping with these plasmids showed that pN419 hybridized to a region covering ca. 490 bases at one end of the genome. One of the other seven cross-hybridizing mRNA band 3 plasmids, pN144, hybridized at ca. 960 to 1,400 bases from the genome end (Table 3). Since the entire length of mRNA 3 is estimated to be 1,420 bases (molecular weight, 4.84×10^5; 11), these hybridization measurements indicate that these two plasmids contain nonoverlapping sequences from the two ends of the N gene. If, as the cloning strategy predicts, the majority of the clones carry the 3′ sequences of the mRNAs, then pN144, representing the seven cross-hybridizing plasmids, contains the 3′ sequences of mRNA 3. The insert of pN419 would then contain the 5′ mRNA band 3 sequences. This could conceivably have originated from a full-length, single-stranded cDNA molecule which, upon 5′ priming, carried out incomplete second-strand synthesis. An analogous clone containing 5′ sequences of the N mRNA has been reported for vesicular stomatitis virus (25).

Examination of the R-loops of pN419 and the viral genome revealed a very short length of unhybridized terminal genomic RNA (Fig. 3A). This may be indicative of a short terminal leader sequence next to the N gene, as in vesicular stomatitis virus (6, 7, 16).

The gene order on the IHNV genome was determined by double R-loop mapping to be N-M1-M2-G-NV-L (Table 4, Fig. 4). With the exception of the NV gene, this order is identical to that of the analogous genes of vesicular stomatitis virus (3′)N-NS-M-G-L (5′) (1, 3) and rabies (3′)N-M1-M2-G-L(5′) (8). Thus, although no 3′ to 5′ orientation was determined directly for the IHNV genome, we assume that the N-ward end is 3′, since an exact inversion of gene order for this rhabdovirus is highly unlikely. In addition, assuming that the cDNA clones contain the 3′ mRNA sequences (with the exception of pN419), then the position of each R-loop agrees with the 3′N to L5′ orientation (Fig. 4). In particular, the hybridization of the pL262 insert to the extreme terminus of the genome, rather than in the middle, implies that the L terminus is the 3′ end of the mRNA sequence, and thus the 5′ end of the gene and genome.

The size of each gene was estimated from the R-loop measurements by assuming that the 5′ boundaries of the R-loops of pN144, pM1163, pM219, pG480, and pL262 corresponded to the 5′ ends of the N, M1, M2, G, NV, and L genes, respectively. These gene sizes are shown in Fig. 4 and agree quite well with the known sizes of the corresponding mRNA species (Fig. 4; 11).

Since the NV gene of IHNV has not been reported to exist in any other rhabdovirus, its position on the genome between the G and L gene was of great interest. Measurements of the pG480 and pNV58 R-loops showed that these sequences were very close and raised the possibility that they might overlap (Table 3). To obtain more accurate measurements, double R-loops of each of these plasmids with pM219, the most proximal gene, were carried out (Fig. 3, Table 4). The gap measurements between M2 and G and between M2 and NV hybridizations showed a better separation of the G and NV R-loops, indicating that they do not overlap but are adjacent. This is supported by the fact that the insert sequences of pG480 and pNV58 do not cross-hybridize. The cDNA insert of pNV58 is 420 base pairs in length (Table 1) and thus represents a minimum of 73% of the mRNA sequence (575 bases; molecular weight, 1.95×10^5; (11). If one excludes a 100-base polyadenylic acid tail from the mRNA length, then this insert could contain as much as 89% of the mRNA coding sequence. This explains the proximity of the R-loop measurements between the pNV58 insert and the pG480 insert from the adjacent G gene. The possibility that the NV and G gene sequences overlap

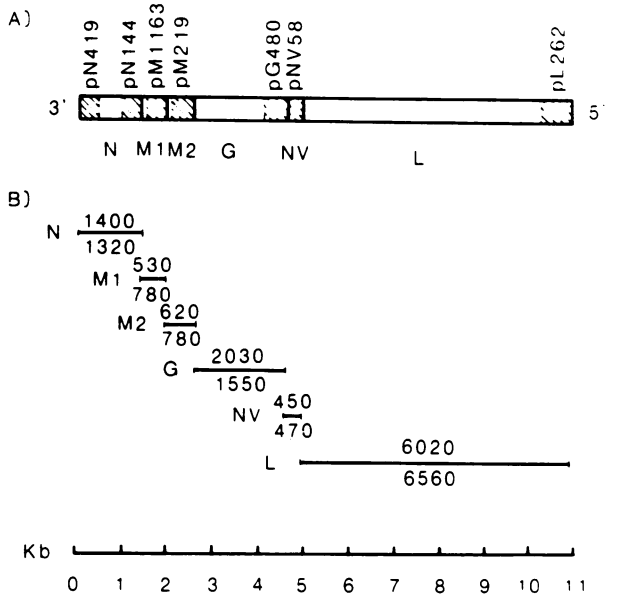

FIG. 4. Physical map of the IHNV genome. (A) Physical map showing the order of genes on the genome (delineated by heavy vertical lines) and locations of cloned sequences (shaded areas) as determined by R-loop measurements (Tables 3 and 4). (B) Horizontal lines represent individual genes. Numerical values (in nucleotides) above each line are gene sizes estimated from R-loop measurements, and values below each line are the corresponding mRNA sizes (11), excluding a polyadenylic acid tail of 100 residues. Kb, kilobases.

also is excluded by the mRNA blot hybridizations and hybrid selection results. If the NV and G genes overlapped, one would expect mRNA blot hybridization of pNV58 to both mRNA bands 2 and 5 and hybrid selection by pNV58 of mRNA for both the NV and G proteins. No such results were seen.

However, the types of data which prove that the NV and G genes do not overlap cannot be used to conclusively rule out the possibility that the NV sequence overlaps, or is contained within, the 3′ end of the L gene. Due to the size of the L gene (Fig. 4) one would not expect hybridization between cDNA sequences from its opposite ends. Hybridization to mRNA band 1 on blots was inconsistent and difficult to detect. The quantity of mRNA 1 in various mRNA preparations was extremely small and variable (11), and its large size (molecular weight, 2.26×10^6; 6.56 kilobases; 11) may have resulted in a lower efficiency of blotting transfer. In addition, the increased problem of degradation of such a large RNA was evident in the high background of lower-molecular-weight hybridization on blots probed with pL232 (Fig. 1f). This inconsistency of blot hybridizations to mRNA 1 and the failure of mRNA 1 to translate in vitro (11) mean that the lack of double hybridization on mRNA blots and double selection during hybrid selection may be due to technical difficulties specific to mRNA 1.

The most straight-forward genetic arrangement is that the NV gene is a separate gene, located between the G and L genes. However, although there is no evidence to indicate that the NV gene sequence does overlap the L sequence, this is an alternative genetic arrangement which has not been ruled out.

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